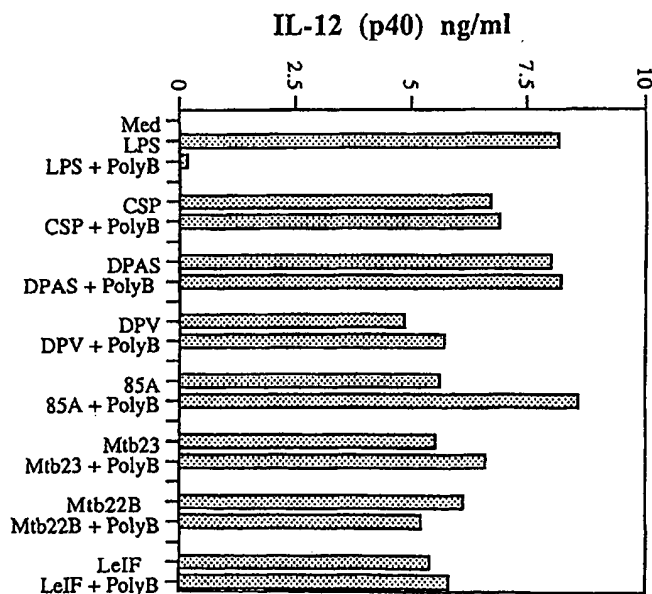




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(54) Title: METHODS FOR USING MYCOBACTERIUM TUBERCULOSIS MOLECULES AS IMMUNOLOGICAL ADJUVANTS



## (57) Abstract

The present invention relates to immunological adjuvants. In particular, it relates to *Mycobacterium tuberculosis* proteins which stimulate interleukin-12 (IL-12) and interferon gamma production. These proteins and fragments thereof may be used in combination with an antigen for inducing and/or enhancing an immune response to the antigen. Alternatively, polynucleotides encoding such proteins may be ligated with an antigen coding sequence to produce a fusion protein as an immunogen.

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METHODS FOR USING  
*MYCOBACTERIUM TUBERCULOSIS*  
MOLECULES AS IMMUNOLOGICAL ADJUVANTS

5

1. INTRODUCTION

10       The present invention relates to immunological adjuvants. In particular, it relates to *Mycobacterium tuberculosis* proteins which stimulate interleukin-12 (IL-12) and interferon-gamma (IFN- $\gamma$ ) production. These proteins and fragments thereof may be used in combination with an antigen for inducing and/or enhancing an immune response to the  
15 antigen. Alternatively, polynucleotides encoding such proteins may be ligated with an antigen coding sequence to produce a fusion protein as an immunogen.

2. BACKGROUND OF THE INVENTION

20

2.1. THE IMMUNE RESPONSE

      The generation of an immune response against an antigen is carried out by a number of distinct immune cell types which interact with each other in the context of the specific antigen. An antigen introduced into the immune system first encounters an antigen  
25 presenting cell. An antigen presenting cell processes the antigen and presents antigenic fragments to helper T cells (TH), which, in turn, stimulate two types of immune responses; i.e., cell-mediated and humoral immune responses. TH respond to antigen stimulation by producing lymphokines which "help" or activate other effector cell types in the immune  
30 system. TH activate B cells to secrete antibodies which function as the major effector molecule in the humoral immune responses. Antibodies neutralize foreign antigens or cooperate with other effector cells in mediating antibody-dependent cellular cytotoxicity. TH regulate cellular immune responses by assisting another T cell subset to develop into  
35 antigen-specific cytotoxic effector cells, which directly kill antigen-positive target cells.

      TH are distinguished from cytotoxic T lymphocytes and B cells by their cell surface expression of a glycoprotein marker termed CD4. In the mouse, TH can be divided into two subsets on the basis of their secretory products. Type 1 helper T cells (TH1) produce interleukin-2 (IL-2) and IFN- $\gamma$  upon activation, while type 2 helper T cells (TH2) produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mossman and Sad, 1996 Immunol. Today). Based on the profile of lymphokine production, TH1 appear to be involved in promoting the

activation and proliferation of other T cell subsets such as cytotoxic T cells, whereas TH2 specifically regulate B cell proliferation and differentiation, antibody synthesis, and antibody class switching.

Cytotoxic T cells express the CD8 surface marker. Unlike most TH, these cells display cytolytic activity upon direct contact with target cells, although they are also capable of producing certain lymphokines. *In vivo*, these cells are particularly important in situations where an antibody response alone is inadequate. There is a preponderance of experimental evidence that cytotoxic T cells rather than B cells and their antibody products play a principal role in the defense against viral infections, intracellular parasitic infections and cancer.

## 2.2. IMMUNOLOGICAL ADJUVANTS

An immunological adjuvant is an agent that enhances the immune response to an antigen. While an immunogen is capable of eliciting an immune response, the magnitude of the response is often weak, thus an adjuvant is added as an additive or vehicle to enhance the response. An adjuvant may function by different mechanisms, including (1) trapping the antigen in the body to cause a slow release, (2) attracting immune cells to migrate to the injection site, (3) stimulating immune cells to proliferate and to become activated, and (4) improving antigen dispersion in the recipient's body.

A number of agents with diverse chemical properties have been used as adjuvants, including oil emulsions, mineral salts, polynucleotides and natural substances. One of the most commonly used adjuvants in laboratory animals is Freund's complete adjuvant, which consists of a water-in-oil emulsion and killed *M. tuberculosis*. Other microorganisms have also been added as immunostimulants to the adjuvant formulation, and they include bacille Calmette-Guerin (an attenuated *Mycobacterium* used as a tuberculosis vaccine in certain countries), *Corynebacterium parvum* and *Bordetella pertussis*. In addition, specific bacterial components have also been included in adjuvant formulations. For example, two bacterial cell wall constituents, lipopolysaccharide and muramyl dipeptide, have been used as adjuvants to stimulate B cells and macrophages, respectively. In humans, alum precipitate is the most widely used adjuvant, which is a suspension of aluminum hydroxide onto which an antigen can be adsorbed.

In vaccine preparation, it is often necessary to mix an immunogen with an adjuvant in order to achieve maximal priming of an immune response. However, many of the most effective adjuvants are toxic to human cells. While bacteria and bacterial components have been used as adjuvants, few defined molecules have been isolated from these sources that provide consistent augmentation of an immune response. Accordingly, there remains a need

for the identification and isolation of molecules that enhance immune responses with minimal toxicities.

### 3. SUMMARY OF THE INVENTION

5       The present invention relates to methods of using *M. tuberculosis* proteins and fragments thereof for inducing and/or enhancing an immune response to an antigen. In particular, it relates to methods of using *M. tuberculosis* secretory proteins to stimulate cytokine production, thereby enhancing antigen-specific and antigen non-specific immune  
10 responses.

      The invention is based, in part, on Applicants' discovery that five secretory proteins of *M. tuberculosis*, designated DPV, DPAS, 85A, Mtb22B and Mtb23, stimulated IL-12 production by macrophages. In addition, DPV also stimulated the production of IFN- $\gamma$ . In  
15 contrast, a large number of other *M. tuberculosis*-derived proteins did not exhibit such cytokine-inducing activities. Since IL-12 induces CD4 T cells to develop into TH1 cells which are particularly important in regulating cytotoxic T cell responses, these five proteins may be used in combination with an antigen to enhance cell-mediated immune responses to the antigen. Furthermore, these proteins may stimulate diverse immune cell types, including  
20 macrophages and natural killer cells.

      It is an object of the invention to prepare a vaccine or therapeutic composition comprising an antigen plus one or more of the molecules disclosed herein for enhancing an  
25 immune response to the antigen.

      It is another object of the invention to prepare a fusion protein between an antigen and one or more of the molecules disclosed herein as an immunogen. Such a protein may be produced by constructing a fusion polynucleotide which is expressed in a host cell followed by protein purification. Alternatively, the polynucleotide may be directly  
30 administered in a subject as a DNA vaccine to induce and/or enhance an immune response.

      It is also an object of the invention to use one or more of the molecules disclosed herein to enhance an immune response in a culture of immune cells. Such cell culture may be used in subsequent adoptive immunotherapy.

35

### 4. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1.       Nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of *M. tuberculosis* antigen DPV.

Figure 2.       Nucleotide sequence (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) of *M. tuberculosis* antigen DPAS.

Figure 3. Recombinant DPV and DPAS proteins stimulated IL-12 production. A total of eighteen *M. tuberculosis*-derived recombinant proteins and a fusion protein of three *M. tuberculosis* antigens (Erd14-DPV-MTI) were incubated with mouse macrophage cell line RAW264.7. The proteins were added at 5 and 20 µg. IL-12 (p40) concentrations were measured by an ELISA.

Figure 4A & B.

Nucleotide sequence (SEQ ID NO:5, Figure 4A) and deduced amino acid sequence (SEQ ID NO:6, Figure 4B) of *M. tuberculosis* antigen 85A.

Figure 5. Nucleotide sequence (SEQ ID NO:7) and deduced amino acid sequence (SEQ ID NO:8) of *M. tuberculosis* antigen Mtb22B. In this figure, Mtb22B is shown as MtbLP22B.

Figure 6. Nucleotide sequence (SEQ ID NO:9) and deduced amino acid sequence (SEQ ID NO:10) of *M. tuberculosis* antigen Mtb23. In this figure, Mtb23 is shown as MtbLP23.

Figure 7. Recombinant *M. tuberculosis* antigens stimulated IL-12 production. The proteins were added at 10µg/ml. Polymixin B was added to inhibit contaminating endotoxin. While polymixin B abrogated the effects of LPS, it did not affect the other molecules.

## **5. DETAILED DESCRIPTION OF THE INVENTION**

### **5.1. ISOLATION OF THE CODING SEQUENCES**

In a specific embodiment by way of example in Section 6, *infra*, the coding sequences for five Mycobacterium molecules were isolated, and their nucleotide and deduced amino acid sequences characterized. In accordance with the invention, any nucleotide sequence which encodes the amino acid sequence of the proteins of the invention can be used to generate recombinant molecules which direct the expression of their coding sequences.

In addition, the invention provides purified polynucleotides containing at least 15 nucleotides (*i.e.*, a hybridizable portion) of a coding sequence; in other embodiments, the

polynucleotides contain at least 25 (continuous) nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of a coding sequence, or a full-length coding sequence. Nucleic acids can be single or double stranded. Such nucleic acids also encode variant forms of the molecule disclosed herein which retain their IL-12 stimulatory activities.

The invention also relates to polynucleotides complementary to the foregoing sequences and polynucleotides hybridizable to such complementary sequences. In a specific embodiment, polynucleotides are provided which comprise a sequence complementary to at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a sequence. In another embodiment, a polynucleotide which is hybridizable to a DPV (SEQ ID NO: 1), DPAS (SEQ ID NO: 3), 85A (SEQ ID NO:5), Mtb22B (SEQ ID NO:7) or Mtb23 (SEQ ID NO:9) coding sequence or its complementary sequence under conditions of low stringency is provided. By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10<sup>6</sup> cpm <sup>32</sup>P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and re-exposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

In another specific embodiment, a polynucleotide which is hybridizable to a coding sequence or its complementary sequence under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10<sup>6</sup> cpm of <sup>32</sup>P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA.

This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

In another specific embodiment, a polynucleotide which is hybridizable to a coding sequence or its complementary sequence under conditions of moderate stringency is provided. For example, but not limited to, procedures using such conditions of moderate stringency are as follows: Filters containing DNA are pretreated for 6 h at 55°C in a solution containing 6X SSC, 5X Denhart's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10<sup>6</sup> cpm <sup>32</sup>P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1X SSC and 0.1% SDS. Filters are blotted dry and exposed for autoradiography. Other conditions of moderate stringency which may be used are well-known in the art. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.1% SDS.

Polynucleotides hybridizable to a complementary sequence of a coding sequence may be naturally-occurring or products of *in vitro* mutagenesis. For the practice of the invention, these polynucleotides encode protein products that stimulate IL-12 and/or IFN-γ production.

## 5.2. POLYPEPTIDES ENCODED BY THE CODING SEQUENCES

In accordance with the invention, a polynucleotide which encodes a polypeptide of the invention, a mutant polypeptide, peptide fragments thereof, fusion proteins containing one or more of them and a heterologous polypeptide, or functional equivalents thereof, may be used to generate recombinant nucleic acid molecules that direct the expression of the protein, peptide fragments, fusion proteins or a functional equivalent thereof, in appropriate host cells.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used in the practice of the invention for the expression of the proteins. Altered DNA sequences which may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a sequence that encodes the same or a functionally equivalent gene product. Additional residues may be added to the amino or carboxyl terminus or both without affecting the activities of a gene product. The gene product itself may contain deletions, additions or substitutions of amino acid residues within a sequence, which result in a silent change thus producing a functionally equivalent protein. Such conservative amino acid substitutions may be made on the basis of similarity

in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine, histidine and arginine; amino acids with uncharged polar head groups having similar hydrophilicity values include  
5 the following: glycine, asparagine, glutamine, serine, threonine, tyrosine; and amino acids with nonpolar head groups include alanine, valine, isoleucine, leucine, phenylalanine, proline, methionine, tryptophan.

The DNA sequences of the invention may be engineered in order to alter a coding  
10 sequence for a variety of ends, including but not limited to, alterations which modify processing and expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, phosphorylation, etc.

In an alternate embodiment of the invention, the coding sequence of the protein  
15 could be synthesized in whole or in part, using chemical methods well known in the art. See, e.g., Caruthers *et al.*, 1980, *Nuc. Acids Res. Symp. Ser.* 7:215-233; Crea and Horn, 180, *Nuc. Acids Res.* 9(10):2331; Matteucci and Caruthers, 1980, *Tetrahedron Letter* 21:719; and  
20 Chow and Kempe, 1981, *Nuc. Acids Res.* 9(12):2807-2817. Alternatively, the protein itself could be produced using chemical methods to synthesize an amino acid sequence in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography. (See  
25 Creighton, 1983, *Proteins Structures And Molecular Principles*, W.H. Freeman and Co., N.Y. pp. 50-60). The composition of the synthetic polypeptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; see Creighton, 1983, *Proteins, Structures and Molecular Principles*, W.H. Freeman and Co., N.Y., pp. 34-49).

30 The production and use of derivatives and analogs related to the proteins disclosed herein are also within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting one or more functional activities associated with a full-length, wild-type protein. As one example, such derivatives  
35 or analogs stimulate IL-12 and/or IFN- $\gamma$  production.

In a specific embodiment of the invention, a polypeptide containing at least 10 (continuous) amino acids is provided. In other embodiments, the polypeptide may contain at least 20 or 50 amino acids of the proteins disclosed herein. In specific embodiments, such polypeptides do not contain more than 100, 150 or 200 amino acids. Derivatives or analogs include but are not limited to molecules comprising regions that are substantially homologous to the proteins of the invention or fragments thereof (e.g., in various

embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or product encoded by a polynucleotide is capable of hybridizing to a naturally-occurring coding sequence, under  
5 highly stringent, moderately stringent, or low stringent conditions.

The derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the nucleic acid or protein level. For example, a cloned coding sequence can be modified by  
10 any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the  
15 production of a polynucleotide encoding a derivative or analog, care should be taken to ensure that the modified coding sequence remains within the same translational reading frame as the native sequence, uninterrupted by translational stop signals, in the gene region where the desired biologic activity is encoded.

20 Additionally, the coding sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed  
25 mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), and the like.

Manipulations may also be made at the protein level. Included within the scope of the invention are protein fragments or other derivatives or analogs which are differentially  
30 modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cytokine or an antigen. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by  
35 cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH<sub>4</sub>, acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives can be chemically synthesized. For example, a peptide corresponding to a portion of a protein disclosed herein which comprises the desired domain or which mediates the desired cytokine stimulatory activities, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the sequence. Non-

classical amino acids include but are not limited to the D-isomers of the common amino acids,  $\alpha$ -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid,  $\gamma$ -Abu,  $\epsilon$ -Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine,  $\beta$ -alanine, fluoro-amino acids, designer amino acids such as  $\beta$ -methyl amino acids,  $C\alpha$ -methyl amino acids,  $N\alpha$ -methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

10 In a specific embodiment, the derivative is a chimeric, or fusion protein containing one or more proteins of the invention or a fragment thereof (preferably consisting of at least a domain or motif of the protein, or at least 10 amino acids of the protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different  
15 protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid  
20 sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of a coding sequence fused to any  
25 heterologous protein-encoding sequences may be constructed.

25 In another specific embodiment, the derivative is a molecule comprising a region of homology with a protein disclosed herein. By way of example, in various embodiments, a first protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%,  
30 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region or when compared to an aligned sequence of the second region that has been aligned by a computer homology  
35 program known in the art.

### 5.3. EXPRESSION SYSTEMS

In order to express a biologically active protein, the nucleotide sequence coding for it, or a functional equivalent, is inserted into an appropriate expression vector, *i.e.*, a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. The gene products as well as host cells or cell lines transfected or transformed with recombinant expression vectors can be used for a variety of purposes.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing a coding sequence for the proteins disclosed herein and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques and *in vivo* recombination/genetic recombination. (See, e.g., the techniques described in Sambrook et al., 1989, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y.)

A variety of host-expression vector systems may be utilized to express a coding sequence. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing a coding sequence; yeast transformed with recombinant yeast expression vectors containing a coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing a coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing a coding sequence; or animal cell systems.

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage  $\lambda$ , plac, ptrp, ptac (ptrp-lac hybrid promoter; cytomegalovirus promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedron promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll  $\alpha/\beta$  binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of a coding sequence, SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems a number of expression vectors may be advantageously selected depending upon the use intended for the expressed products. For example, when large quantities of the proteins are to be produced for the generation of antibodies or as therapeutic compositions, vectors which direct the expression of high levels of fusion

protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which a coding sequence may be ligated into the vector in frame with the *lacZ* coding region so that a hybrid protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety. In a preferred embodiment, pET-17b plasmid is used in BL21 pLysE *E. coli* to express proteins with six histidine residues at the amino terminus which can be purified by Ni-NTA agarose affinity columns.

#### 5.4. IDENTIFICATION AND PURIFICATION OF PROTEINS

Once a recombinant protein is expressed, it can be identified by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay, ELISA, bioassays, etc.

Once the encoded protein is identified, it may be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be evaluated using any suitable assay such as stimulation of IL-12 and IFN- $\gamma$  production. For the practice of the present invention, it is preferred that the proteins disclosed herein are at least 80% purified from other proteins. It is more preferred that they are at least 90% purified. For *in vivo* administration, it is preferred that the proteins are greater than 95% purified.

In another alternate embodiment, native proteins can be purified from natural sources, by standard methods such as those described above (*e.g.*, immunoaffinity purification). In a specific embodiment of the present invention, the proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods or by purification of native proteins, include but are not limited to those containing, as a primary amino acid sequence, all or part of the amino acid sequences substantially as depicted in Figures 1, 2, 4, 5 and 6 (SEQ ID NOS: 2, 4, 6, 8 and 10), as well as fragments and other derivatives, and analogs thereof, including proteins homologous thereto.

### 5.5. USES OF THE CODING SEQUENCES

The coding sequence for the proteins disclosed herein may be used to encode a protein product for use as an immunological adjuvant to induce and/or enhance immune responses to an antigen. In addition, such coding sequence may be ligated with a coding sequence of an antigen to construct a fusion polynucleotide. A fusion polynucleotide may also be used to express a recombinant protein for use as an immunogen. Alternatively, the polynucleotide may be used *in vivo* as a DNA vaccine (United States Patent Nos. 5,589,466; 5,679,647; 5,703,055). In this embodiment of the invention, the polynucleotide expresses its encoded protein in a recipient to directly induce an immune response

In a preferred embodiment, a therapeutic composition comprises a coding sequence that is part of an expression vector that also encodes an immunogenic protein or a fragment thereof. In particular, such a polynucleotide contains a promoter operably linked to the coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another embodiment, a polynucleotide contains a coding sequence flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the coding sequence (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

Delivery of the nucleic acid into a subject may be either direct, in which case the subject is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the subject. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene transfer.

In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (see U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) which can be used to target cell types specifically expressing the receptors, etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another

embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180 dated April 16, 1992; WO 92/22635 dated December 23, 1992; WO92/20316 dated November 26, 1992; WO93/14188 dated July 22, 1993; WO 93/20221 dated October 14, 1993). Alternatively,  
5 the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

In a specific embodiment, a viral vector such as a retroviral vector can be used (see  
10 Miller et al., 1993, Meth. Enzymol. 217:581-599). Retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. An adjuvant protein coding sequence and an antigen coding sequence are cloned into the vector, which facilitates delivery of the genes into a recipient.  
15 More detail about retroviral vectors can be found in Boesen et al., 1994, Biotherapy 6:291-302, which describes the use of a retroviral vector to deliver the *mdr1* gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., 1994, J. Clin. Invest. 93:644-651; Kiem et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993,  
20 Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses  
25 naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Adeno-associated virus (AAV) has also been proposed for use in *in vivo* gene transfer  
30 (Walsh et al., 1993, Proc. Soc. Exp. Biol. Med. 204:289-300).

Another approach involves transferring a construct to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the  
35 cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a subject.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene

transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention.

5

#### 5.6. USES OF THE POLYPEPTIDES

Purified or partially purified proteins disclosed herein or fragments thereof may be mixed with an antigen for the preparation of a vaccine or therapeutic composition. In addition, such proteins may be further suspended in an oil emulsion to cause a slower release of the proteins *in vivo* upon injection. The optimal ratios of each component in the formulation may be determined by techniques well known to those skilled in the art. For the practice of the invention, an antigen is any molecule that induces an immune response against it. In particular, it includes, but is not limited to, a molecule derived from an infectious agent such as a bacterium, a virus, a fungus, a protozoan and a parasite. In addition, a tumor antigen which is preferentially expressed in certain tumor types is also within the scope of the invention. A tumor antigen may be of cellular origin or encoded by a virus.

Such a formulation may be administered to a subject *per se* or in the form of a pharmaceutical or therapeutic composition. Pharmaceutical compositions comprising the proteins may be manufactured by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries which facilitate processing of the protein or biologically active peptides into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For topical administration, the proteins may be formulated as solutions, gels, ointments, creams, suspensions, etc. as are well-known in the art.

Systemic formulations include those designed for administration by injection, *e.g.* subcutaneous, intravenous, intramuscular, intrathecal or intraperitoneal injection, as well as those designed for transdermal, transmucosal, oral or pulmonary administration.

For injection, the proteins may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the proteins may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, a composition can be readily formulated by combining the proteins with pharmaceutically acceptable carriers well known in the art. Such carriers  
5 enable the proteins to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. For oral solid formulations such as, for example, powders, capsules and tablets, suitable excipients include fillers such as sugars, such as lactose, sucrose, mannitol and sorbitol; cellulose preparations  
10 such as maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP); granulating agents; and binding agents. If desired, disintegrating agents may be added, such as the cross-linked polyvinylpyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

15 If desired, solid dosage forms may be sugar-coated or enteric-coated using standard techniques.

For oral liquid preparations such as, for example, suspensions, elixirs and solutions, suitable carriers, excipients or diluents include water, glycols, oils, alcohols, etc.  
20 Additionally, flavoring agents, preservatives, coloring agents and the like may be added.

For buccal administration, the proteins may take the form of tablets, lozenges, etc. formulated in conventional manner.

For administration by inhalation, the proteins for use according to the present  
25 invention are conveniently delivered in the form of an aerosol spray from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to  
30 deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The proteins may also be formulated in rectal or vaginal compositions such as suppositories or retention enemas, e.g, containing conventional suppository bases such as  
35 cocoa butter or other glycerides.

In addition to the formulations described previously, the proteins may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the proteins may be formulated with suitable polymeric or hydrophobic

materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Alternatively, other pharmaceutical delivery systems may be employed. Liposomes and emulsions are well known examples of delivery vehicles that may be used to deliver an antigen and an adjuvant protein disclosed herein. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the proteins may be delivered using a sustained-release system, such as semipermeable matrices of solid polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the proteins for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

Determination of an effective amount of the proteins disclosed herein for administration is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein.

An effective dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models to achieve an induction or enhancement of an immune response using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data. Dosage amount and interval may be adjusted individually. Typically, a pharmaceutical or vaccine composition contains an adjuvant polypeptide in the range of 1 µg to 5mg in a volume of 0.1 to 5 ml. The amount of each protein administered will, of course, be dependent on the subject being treated, on the subject's weight, the manner of administration and the judgment of the physician.

The invention having been described, the following examples are offered by way of illustration and not limitation.

## 6. EXAMPLE: MYCOBACTERIUM TUBERCULOSIS SECRETORY PROTEINS STIMULATE PRODUCTION OF CYTOKINES

### 6.1. MATERIALS AND METHODS

#### 6.1.1. PRODUCTION OF RECOMBINANT PROTEINS

Recombinant proteins were expressed in *E. coli* with six histidine residues at the amino-terminal portion using the pET plasmid vector (pET-17b) and a T7 RNA polymerase

expression system (Novagen, Madison, WI). *E. coli* strain BL21 (DE3) pLysE (Novagen) was used for high level expression.

The recombinant (His-Tag) antigens were purified from the soluble supernatant or the insoluble inclusion body of 500 ml of IPTG induced batch cultures by affinity chromatography using the one step QIAexpress Ni-NTA agarose matrix (QIAGEN, Chatsworth, CA) in the presence of 8M urea. Briefly, 20 ml of an overnight saturated culture of BL21 containing the pET construct was added into 500 ml of 2xYT media containing 50 µg/ml ampicillin and 34 µg/ml chloramphenicol, grown at 37°C with shaking. The bacterial cultures were induced with 2mM IPTG at an OD<sub>560</sub> of 0.3 and grown for an additional 3 h (OD = 1.3 to 1.9). Cells were harvested from 500 ml batch cultures by centrifugation and resuspended in 20 ml of binding buffer (0.1 M sodium phosphate, pH 8.0; 10 mM Tris-HCl, pH 8.0) containing 2mM PMSF and 20 µg/ml leupeptin plus one complete protease inhibitor tablet (Boehringer Mannheim) per 25 ml. *E. coli* was lysed by freeze-thaw followed by brief sonication, then spun at 12k rpm for 30 min. to pellet the inclusion bodies.

The inclusion bodies were washed three times in 1% CHAPS in 10 mM Tris-HCl (pH 8.0). This step greatly reduced the level of contaminating LPS. The inclusion body was finally solubilized in 20 ml of binding buffer containing 8 M urea or 8 M urea was added directly into the soluble supernatant. Recombinant antigens with His-Tag residues were batch bound to Ni-NTA agarose resin (5 ml resin per 500 ml inductions) by rocking at room temperature for 1 h and the complex passed over a column. The flow through was passed twice over the same column and the column washed three times with 30 ml each of wash buffer (0.1 M sodium phosphate and 10 mM Tris-HCl, pH 6.3) also containing 8 M urea. Bound protein was eluted with 30 ml of 150 mM imidazole in wash buffer and 5 ml fractions collected. Fractions containing each recombinant antigen were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0) bound one more time to the Ni-NTA matrix, eluted and dialyzed in 10 mM Tris-HCl (pH 7.8). The yield of recombinant protein varied from 25-150 mg per liter of induced bacterial culture with greater than 98% purity. Recombinant proteins were assayed for endotoxin contamination using the *Limulus* assay (BioWhittaker) and were shown to contain <10 E.U./mg.

#### 6.1.2. ANIMALS AND CELL LINES

C3H SCID mice were maintained at the animal facilities. RAW264 cell line was maintained in RPMI containing 10% FBS, 20µM 2-mercaptoethanol and 50 µg/ml gentinimicin. One ml of cells (at a density of  $5 \times 10^5$  cells per ml) was plated per well using a 24 well plate format and allowed to adhere overnight. The media and nonadherent cells were removed by aspiration and the cells replenished with 1.0 ml of media containing 1.2%

DMSO and allowed to differentiate for 8 hr. The cells were subsequently activated with rIFN- $\gamma$  (20 ng/ml; R&D Lot#RY 147101) and incubated for about 14 hrs (overnight). Recombinant proteins were added at the desired concentration (usually 20  $\mu$ g/ml) in the presence or absence of 10  $\mu$ g/ml polymixin B. Lipopolysaccharide (LPS) was used as a  
5 positive control at a concentration of 1  $\mu$ g/ml.

#### 6.1.3. IL-12 ASSAY

ELISA plates (Corning) were coated with 50  $\mu$ l/well of 5  $\mu$ g/ml anti-mouse IL-12  
10 monoclonal antibody C17.15.10.12, in 0.1 M bicarbonate coating buffer, pH9.6 and incubated for 4 hr at room temperature. Plate contents were shaken out and blocked with PBS-0.05% Tween, 1.0% BSA (200  $\mu$ l/well) overnight at 4°C and washed for 6 times in PBS-0.1% Tween. Standards (recombinant mouse IL-12 p40) and supernatant samples from  
15 the stimulated RAW 264 cells were diluted in PBS-0.05% Tween, followed by the addition of 0.1% BSA and incubated overnight at 4°C. The plates were washed as above and then incubated with 100  $\mu$ l/well of the biotinylated second anti-mouse IL-12 antibody (C15.6.7.6) at 0.5  $\mu$ g/ml diluted in PBS-0.1% Tween, 0.1% BSA and incubated for 1 hr at room temperature. After washing, plates were incubated with 100  $\mu$ l/well of streptavidin-HRP  
20 (Zymed) at a 1:2500 dilution in PBS-0.05% Tween, 0.1% BSA at room temperature for 1 hr. The plates were washed one last time and developed with 100  $\mu$ l/well TMB substrate (3,3',5,5' - tetramethylbenzidine, Kirkegaard and Perry, Gaithersburg, MD) and the reaction stopped after color developed, with H<sub>2</sub>SO<sub>4</sub> at 50  $\mu$ l/well. Absorbance (OD) were determined  
25 at 450 nm using 570 nm as a reference wavelength and the cytokine concentration evaluated using the standard curve.

#### 6.1.4. IFN- $\gamma$ ASSAY

30 Spleens from C3H/HeJ SCID mice were removed aseptically and single cell suspension prepared in complete RPMI following lysis of red blood cells. 100  $\mu$ l of cells (2x10<sup>5</sup> cells) were plated per well in a 96-well flat bottom microtiter plate. Cultures were stimulated with recombinant proteins for 24h and the supernatant assayed for IFN- $\gamma$ .

35 The levels of supernatant IFN- $\gamma$  were analyzed by sandwich ELISA, using antibody pairs and procedures available from PharMingen. Standard curves were generated using recombinant mouse cytokines. ELISA plates (Corning) were coated with 50  $\mu$ l/well (1  $\mu$ g/ml, in 0.1 M bicarbonate coating buffer, pH9.6) of a cytokine capture rat anti-mouse IFN- $\gamma$  monoclonal antibody (PharMingen; Cat. # 18181D), and incubated for 4 hr at room temperature. Plate contents were shaken out and blocked with PBS-0.05% Tween, 1.0% BSA (200  $\mu$ l/well) overnight at 4°C and washed for 6 times with PBS-0.1% Tween.

Standards (recombinant mouse IFN- $\gamma$ ) and supernatant samples diluted in PBS-0.05% Tween, 0.1% BSA were then added for 2 hr at room temperature. The plates were washed as above and then incubated for 2 hr at room temperature with 100  $\mu$ l/well of a second biotinylated rat anti-mouse IFN- $\gamma$  antibody (Cat. # 18112D; PharMingen) at 0.5  $\mu$ g/ml diluted in PBS-0.05% Tween, 0.1% BSA. After washing, plates were incubated with 100  $\mu$ l/well of streptavidin-HRP (Zymed) at a 1:2500 dilution in PBS-0.05% Tween, 0.1% BSA at room temperature for 1 hr. The plates were washed one last time and developed with 100  $\mu$ l/well TMB substrate (3,3',5,5' — tetramethylbenzidine, Kirkegaard and Perry, Gaithersburg, MD) and the reaction stopped after color developed, with H<sub>2</sub>SO<sub>4</sub>, 50  $\mu$ l/well. Absorbance (OD) were determined at 450 nm using 570 nm as a reference wavelength and the cytokine concentration evaluated using the standard curve.

## 6.2. RESULTS

A number of molecularly cloned *M. tuberculosis* antigens were expressed as recombinant proteins in *E. coli*. After purification, these proteins were tested for their ability to stimulate the production of interleukin-12 (IL-12) by a mouse macrophage cell line, RAW264. The concentrations of IL-12 in RAW264 culture supernatants were measured by an ELISA assay using an antibody to detect the p40 component of the IL-12 heterodimer.

Among the eighteen proteins tested, only two, designated DPV (SEQ ID NO:2) (Figure 1) and DPAS (SEQ ID NO:4) (Figure 2) stimulated the production of IL-12 (Figure 3). The stimulatory activities of these proteins were not affected by the inclusion of polymyxin B in the cultures, which abrogated the IL-12 stimulatory activities of lipopolysaccharide, a polyclonal B cell mitogen. Both DPV and DPAS are secretory proteins of *M. tuberculosis*.

A polynucleotide was constructed to ligate the coding sequences of three *M. tuberculosis*-derived antigens into a fusion sequence. The fusion product (ErD14-DPV-MTI) encoded by such polynucleotide also exhibited IL-12 stimulatory activities. Among the three antigens contained in the fusion protein, DPV was the only IL-12-stimulator when used as an individual antigen. Therefore, the inclusion of DPV in a larger fusion protein did not affect its IL-12 stimulatory activities.

Further testing of additional *M. tuberculosis* antigens identified three more proteins as capable of stimulating IL-12 production. These antigens are referred to as 85A (SEQ ID NO:6)(Figure 4A and B), Mtb22B (SEQ ID NO:8)(Figure 5) and Mtb23 (SEQ ID NO:10)(Figure 6). Recombinant 85A, Mtb22B and Mtb23 stimulated the production of IL-12 by RAW264 cells in the presence of polymyxin B (Figure 7).

Additionally, DPV was further tested for its ability to stimulate IFN- $\gamma$  production by splenocytes of C3H SCID mice. DPV induced 12.519 ng/ml of IFN- $\gamma$  as measured by ELISA, whereas total *M. tuberculosis* culture filtrate proteins induced 1.773 ng/ml of IFN- $\gamma$ , as compared with media alone as background. Since SCID mouse splenocytes do not  
5 contain mature T and B cells, the cell source of IFN- $\gamma$  production is presumed to be natural killer cells. Thus, DPV stimulates immune cells beyond T cell mediated responses.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and any clones, DNA  
10 or amino acid sequences which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims. It is also to be understood that all base pair sizes given for nucleotides are  
15 approximate and are used for purposes of description.

All publications cited herein are incorporated by reference in their entirety.

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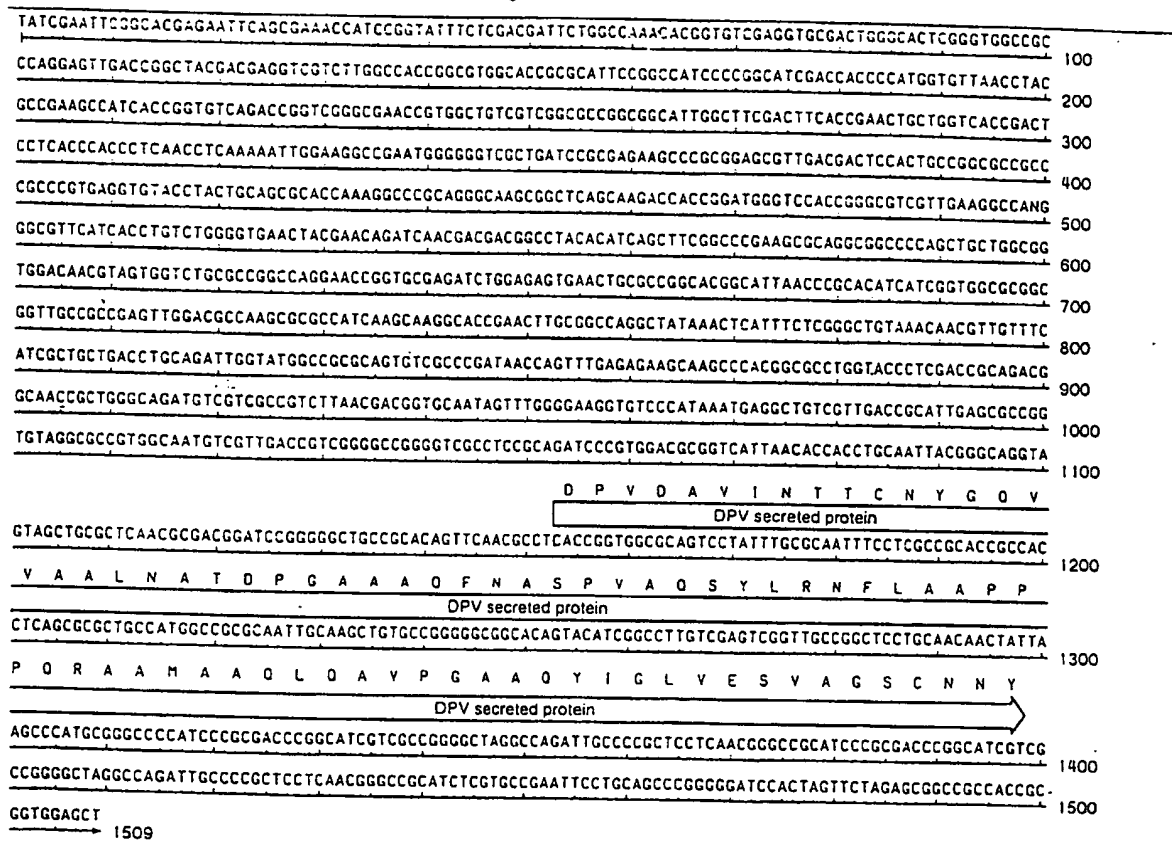
35

WHAT IS CLAIMED IS:

1. A method of enhancing an immune response to an antigen, comprising contacting an immune cell population with an antigen and an isolated protein which comprises the amino acid sequence of any one of SEQ ID NO:2, 4, 6, 8 and 10, or a fragment thereof.
2. A method of enhancing an immune response in a subject to an antigen, comprising administering to the subject an effective amount of an antigen and an isolated protein which comprises the amino acid sequence of any one of SEQ ID NO:2, 4, 6, 8 and 10, or a fragment thereof.
3. A method of enhancing an immune response in a subject to an antigen, comprising administering to the subject an expression vector which comprises a coding sequence for an antigen and a coding sequence for a protein having the amino acid sequence of any one of SEQ ID NO:2, 4, 6, 8 and 10, or a fragment thereof or a combination thereof.
4. A method of enhancing an immune response in a subject to an antigen, comprising administering to the subject an expression vector which comprises a coding sequence for an antigen, and a second expression vector which comprises a coding sequence for a protein having the amino acid sequence of any one of SEQ ID NO:2, 4, 6, 8 or 10, or a fragment thereof or a combination thereof.
5. A therapeutic composition comprising an antigen and a protein which comprises the amino acid sequence of any one of SEQ ID NO:2, 4, 6, 8 or 10, or a fragment thereof.
6. A therapeutic composition comprising an expression vector comprising an antigen coding sequence and a coding sequence for a protein having the amino acid sequence of any one of SEQ ID NO:2, 4, 6, 8 or 10, or a fragment thereof.

9532-030 (sheet 1 of 7)

Figure 1



9532-030 (Sheet 2 of 7)

Figure 2

AGAAATAATTTTGTAACTTTAAGAAGGAGATATACATATGCATCACCATCACCATCACACCGGTAGTTTGAACCAACGCACAATCGACGGGCAAAACG 100

AACGGAAGAACAACAACCATGAAGATGGTGAATCGATCGCCGCAGGTCTGACCGCCCGCGGCTGCAATCGGCGCCGCTGCGGCCGGTGTGACTTCGATCAT 200

GGCTGGCGGCCCGGTCTGATACCAGATGCAGCGGTCTGCTTCGGCGGCCCACTGCCGTTGGAACCCGGCATCCGCCCTGACGTCCCGACCGCCGCCAG 300

DPAS secreted protein

TTGACCAGCCTGCTCAACAGCCTCGCCGATCCCAACGTGTCGTTTGGCAACAAGGGCAGTCTGGTCGAGGGCGGCATCGGGGCAACCGAGGCGCGCATCG 400

LTSLNSLADPNVVSFANKGSLVECGIGGTEARI

DPAS secreted protein

CCGACCACAAGCTGAAGAAGGCCCGGAGCACGGGGATCTGCCGCTGTCGTTTACGCGTGACGAACATCCAGCCGGCGGCCCGGTTCCGGCCACCGCCGA 500

ADHKLKXAAEHGDLPLSFSTNTIQPAAAGSATAO

DPAS secreted protein

CGTTTCGCTCGGGTCCGAAGCTCTGTCGCCGGTCACGCAGAACGTACGTTCTGTGAATCAAGGCGGCTGGATGCTGCACGCGCATCGCGATGGAG 600

VSVSGPKLSSPVTONVTFFVNOGGWMLSRASAME

DPAS secreted protein

TTGCTGCAGGCGCAGGGAAGTATTGGCGGCCCGCTTCAGCCCGCTGTTACGTACGCCCGCCGCTGGTGACGCGTCCATGTGAACACTCGCGCGT 700

LLQAAAGN

DPAS secreted protein

GTAGCACGGTGGGTTCCGCAGGGCCGCACGCACGCCCGGTGCAAGCCGCTCTCGAGATAGGTGGTGCCTCGCCACCAGCGACACCCCGCCCTCGCC 800

TGTTCTCGTCTGCATGAGTTCGTGCACGTGGTGTGCACACCGAGGGCTTGTGTACGTGATCGCCGATGCTCATCCGAGCACCCAGGACCGATCGCT 900

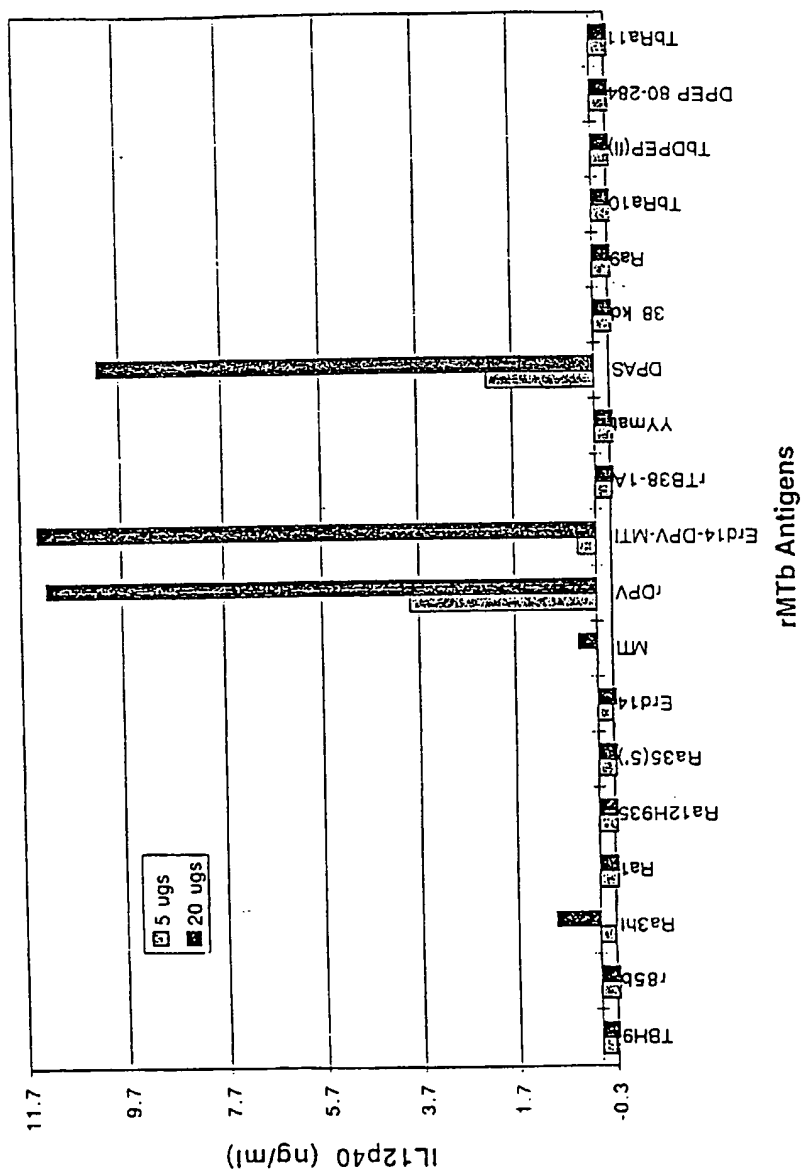
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TCTACCGACAGCGCCGCCGAGTTCATCGCGGTCACGAGGACGAGGCCGAACGGCTCCTCGTGCCGAATTCTGCAGATATCCATCACACTGGCGGCC 1200

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Figure 3  
9532-030 (sheet 3 of 7)



9532-030 (sheet 4 of 7)

Figure 4A

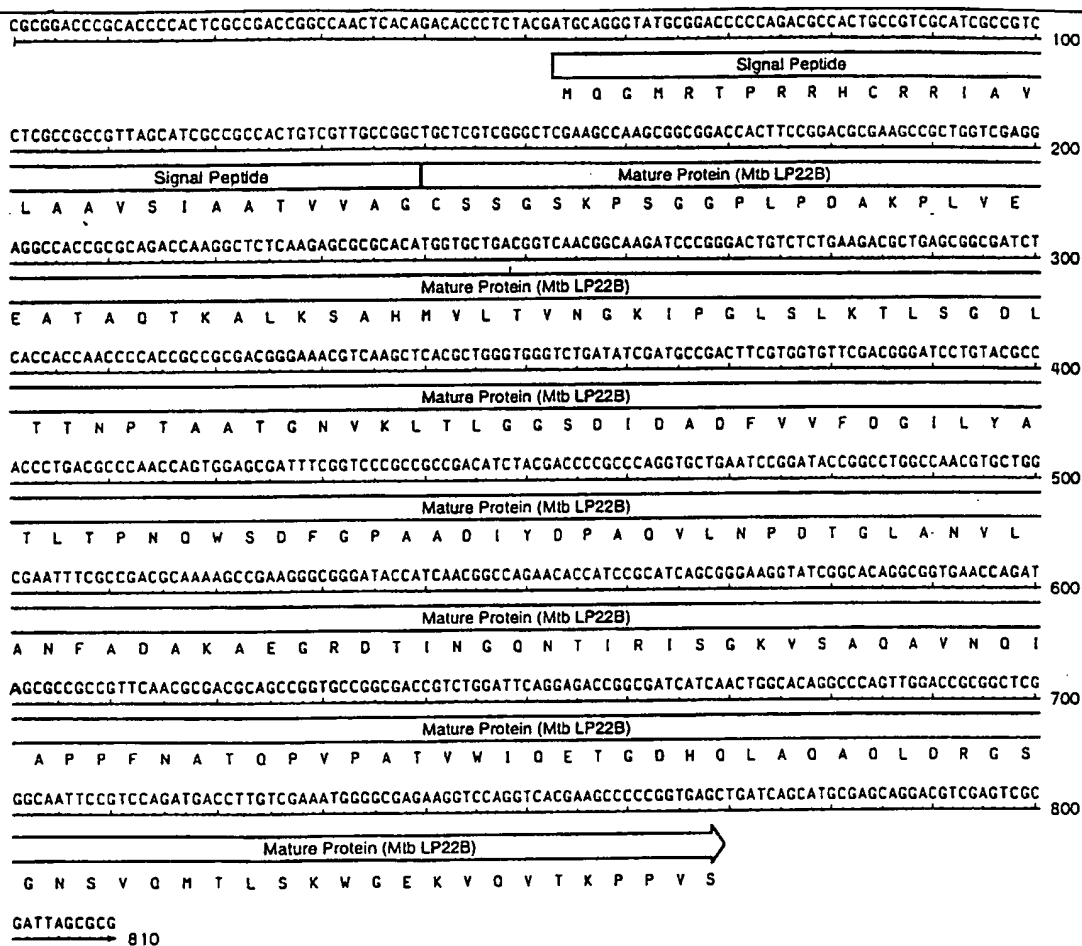
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AGTGGTGGTGCCAACTCGCCCGCCCTGTACCTGCTCGACGGCCTGCGCGCGC  
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CCGACTGGTACCAGCCCGCCTGCGGCAAGGCCGGTTGCCAGACTTACAAGTG  
GGAGACCTTCCTGACCAGCGAGCTGCCGGGTGGCTGCAGGCCAACAGGCAC  
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GCTGACGCTGGCGATCTATACCCCCAGCAGTTCGTCTACGCGGGAGCGATG  
TCGGGCCTGTTGGACCCCTCCCAGGCGATGGGTCCCACCCTGATCGGCCTGG  
CGATGGGTGACGCTGGCGGCTACAAGGCCTCCGACATGTGGGGCCCGAAGG  
AGGACCCGGCGTGCGAGCGCAACGACCCGCTGTTGAACGTCGGGAAGCTGAT  
CGCCAACAACACCCCGCTCTGGGTGTACTGCGGCAACGGCAAGCCGTCGGAT  
CTGGGTGGCAACAACCTGCCGGCCAGGTTCTCGAGGGCTTCGTGCGGACCA  
GCAACATCAAGTTCCAAGACGCCTACAACGCCGGTGGCGGCCACAACGGCGT  
GTTGACTTCCCGGACAGCGGTACGCACAGCTGGGAGTACTGGGGCGCGCAG  
CTCAACGCTATGAAGCCCGACCTGCAACGGGCACTGGGTGCCACGCCCAACA  
CCGGGCCCCGCGCCCCAGGGCGCCTAGCTCCGAACAGACGAATTC

MHHHHHHSRPLPVEYLQVPSMGRDIKVQFQSGGANSALYLLDGLRAQD  
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TFLTSELPGLQANRHVKPTGSAVVGLSMAASSALTIAIYHPQQFVYAGAMSGL  
LDPSQAMGPTLIGLAMGDAGGYKASDMWGPKEPAWQRNDPLLNVGKLIANN  
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Figure 4B

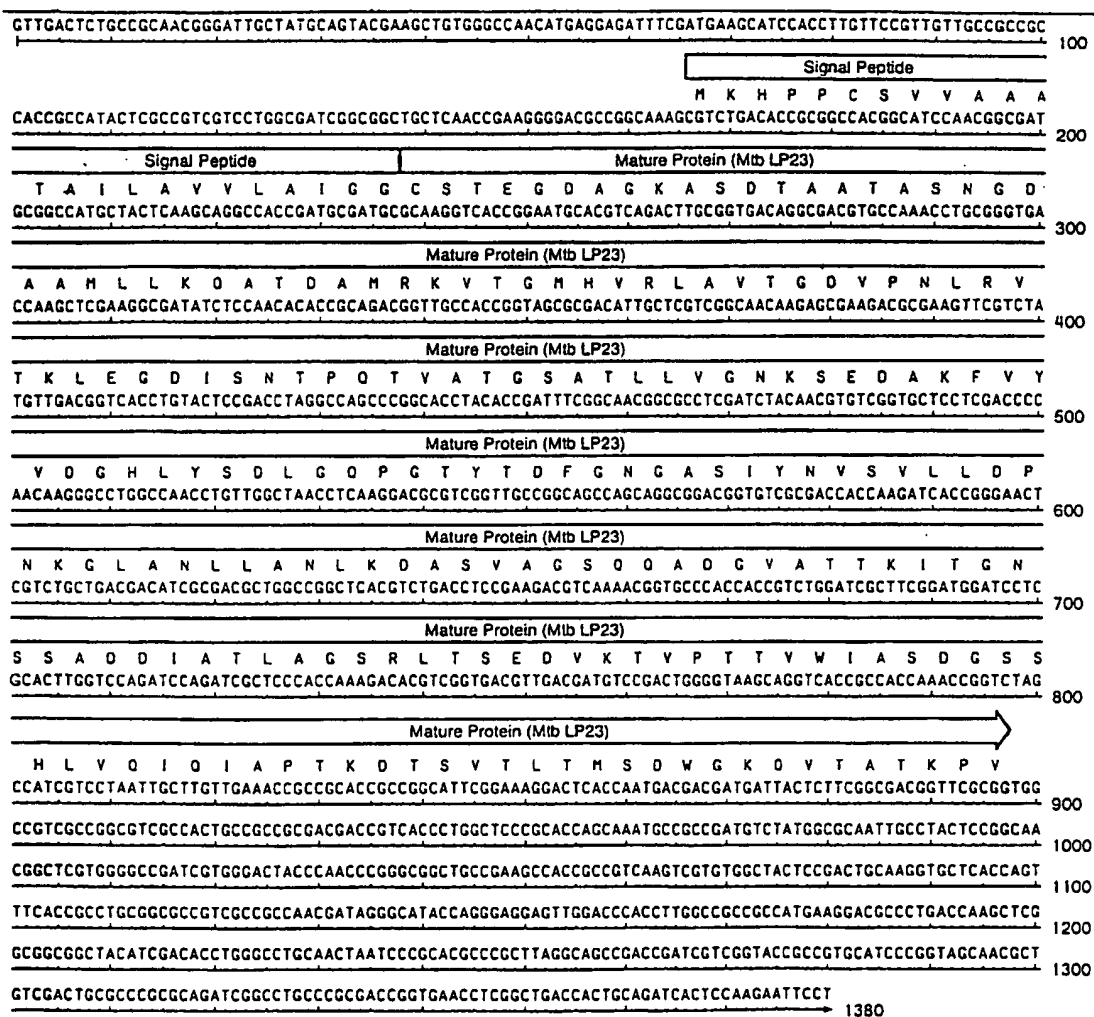
9532-030 (sheet 5 of 7)

Figure 5



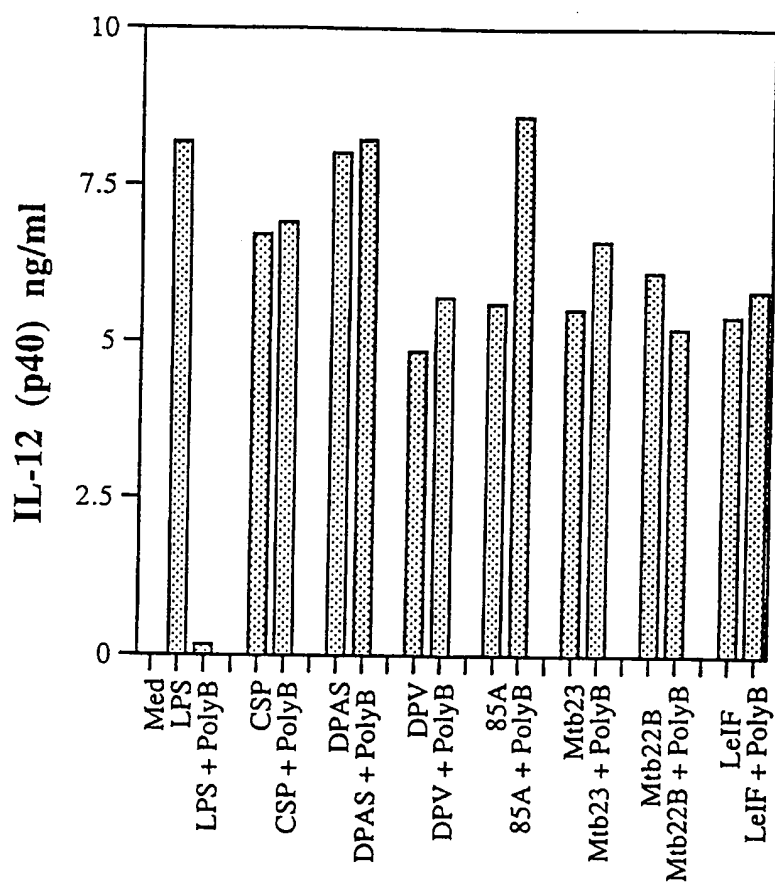
9532-030 (sheet 6 of 7)

Figure 6



9532-030 (sheet 7 of 7)

Figure 7



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